# THE GRAM-NEGATIVE BACTEROIDES OF THE INTESTINE

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Received for publication November 3, 1936

In 1933 Eggerth and Gagnon published a paper entitled "The Bacteroides of Human Feces." The results presented were illuminating in that they aided materially in the opening of a new field in intestinal bacteriology and broadened existing knowledge concerning non-sporulating anaerobes.

Eggerth and Gagnon state that in 91 per cent of a series of 60 stools of adults the predominating organisms were obligate anaerobes of the genus *Bacteroides*. They isolated and made a taxonomic study of Gram-negative *Bacteroides*.

Eggerth and Gagnon's observations naturally lead one to assume that these organisms must play a rôle of prime importance in the intestine, and that they constitute valuable material for further intensive investigation. No further stimulus was required for the present writers to enter this new field.

The following were points of outstanding interest: (1) the relative numbers of bacteroides in human feces; (2) the proportion of Gram-negative to Gram-positive non-sporulating anaerobes; (3) a comprehensive morphological, cultural and serological study, and (4) the adoption of a definite system of classification of the isolated strains.

Over 40 stools of human adults were cultured aerobically and anaerobically. A total of 87 anaerobic strains[2] was isolated. To this number were added 22 strains received from Eggerth. These 109 organisms were studied systematically, together with

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[2] Of these, 4 were isolated by K. H. Lewis of this laboratory.

seven strains of *Lactobacillus bifidus*, four of *Lactobacillus acidophilus* and 1 of *Lactobacillus bulgaricus*. Of the 109 different so-called *Bacteroides* strains, 36 were Gram-positive and 73 Gram-negative. The Gram-negative organisms will be dealt with in this paper.

## TECHNIC

The methods and media used by Eggerth and Gagnon were adopted, with a few slight changes. Quantitative determinations were not attempted. The common procedure consisted in making saline suspensions of the feces having an approximate nephelometer reading of 5.0 (MacFarland). With this as dilution 1, 9 further dilutions were made, the dilution being doubled each time. Platings were made on blood agar from dilutions 3 to 10, inclusive, and duplicate plates incubated aerobically and anaerobically.

The basal medium was the one employed by Eggerth and Gagnon, namely beef infusion agar containing 1.5 per cent agar, 1 per cent peptone, and 0.4 per cent disodium phosphate; the final pH was 7.6 to 7.8. At the time of the pouring of the plates 7 per cent defibrinated sterile cows' blood and 0.15 per cent sterile glucose solution were added. In accordance with the observations of Eggerth and Gagnon, an acid reaction was found to be unfavorable to the initial growth of these forms; however, after the organisms were obtained in pure culture, a slight acidity proved to be the most favorable for nearly all strains.

The blood agar plates were incubated usually at 37.5°C. for periods varying from 1 week to 10 days. Anaerobic methods which depend entirely on evacuation were very unsatisfactory due to the drying out of the plates. A procedure was employed (Weiss and Spaulding, 1937) which involves evacuation and filling the jar with tank hydrogen, and the use of palladinized asbestos for the complete removal of the free oxygen. This method proved very satisfactory.

In the isolation of pure cultures, inoculum from selected colonies was streaked on new blood agar plates, and the plates incubated under the usual anaerobic conditions. The process of

re-plating was continued until there was every indication of the plates containing the organism in pure form. This was followed by spore tests. All organisms which were non-spore-forming obligate anaerobic rods were inoculated into egg-meat and held for further study.

Eggerth and Gagnon kept most of their stock cultures of Gram-negative *Bacteroides* strains in brain medium. As this is rather specialized and expensive tissue, and as our laboratory carries the ordinary egg-meat medium regularly, the latter was tried out. All of the strains of Gram-negative and Gram-positive non-sporulating anaerobes appeared to grow well and retain their viability in it; it was adopted, therefore, as the standard stock medium.

The *Bacteroides* group studied here is non-proteolytic. On opening anaerobic jars containing them, a strong offensive odor pervades the laboratory. However, at no time were there any signs of digestion in the egg-meat tubes except when they were contaminated with anaerobic spore-forming bacteria. To avoid contamination from wet plugs, all tubes and plates were covered with paper towels. The members of this group remained alive for 3 months or longer in the egg-meat tubes, at both room and ice box temperatures.

Blood agar and egg-meat are not entirely satisfactory media. The ideal medium would be one that contains all of the required nutrient substances and is sufficiently clear to permit an unobscured view of the bacterial growth. This goal is being sought by another member of this laboratory who is engaged in a study of the group from angles somewhat different from those discussed here.

All special media, such as carbohydrates, milk, gelatin, tryptophane broth, lead acetate and nitrate broth were made according to the directions of Eggerth and Gagnon.

### RELATIVE NUMBERS OF BACTEROIDES IN STOOLS

Our results bear out those of Eggerth and Gagnon, in general. The majority of fecal samples examined revealed the predominating organisms to be members of the *Bacteroides* group. In

many instances *Bacteroides* colonies occurred on the agar plates in apparently pure form, in the higher dilutions of feces. As a rule the best isolations were obtained between the fifth and sixth dilutions; or, between the second and the fifth after the last tube showing visible turbidity. At no time were spore-forming anaerobes observed in large enough numbers to appear in any of the higher dilutions. *Escherichia coli* was the only organism which survived the elimination process, and then only occasionally.

It is but natural to assume that any organism which appears in the intestine in as high concentrations as these non-sporulating anaerobes are found to do, must play some important rôle in the intestine. Such an assumption alone should be a strong stimulus to prosecute intensive search into their nature, food requirements, activities and exact relations to other bacteria and to the host.

Eggerth and Gagnon included both the Gram-negative and Gram-positive non-sporulating anaerobes of the intestine in the so-called genus, *Bacteroides*, although they are apparently open-minded. Because the Gram stain is generally regarded as significant in any classification scheme the present report is devoted to the Gram-negative group, with the intention of making the Gram-positive division the subject of a future paper. This paper then deals with a study of 73 Gram-negative *Bacteroides* strains, 15 of which were obtained from Eggerth and Gagnon, and the remainder isolated in our laboratory from the feces of presumably normal human adults.

What constitutes the *Bacteroides* group or genus? Is it an entity in itself, or is it a group of organisms which is too inclusive, and should be split up into definite and distinct genera? The Gram-negative representatives may be set apart for the moment from all other known organisms, except perhaps the fusiform group, by the fact that they are definitely non-spore-forming obligate anaerobes which do not retain Gram violet stain. There have been numerous references in the literature to Gram-negative non-spore-forming anaerobic rods, but until we can obtain a better description of them, all such forms must be classified under

the head of *Bacteroides* or *Fusobacterium*. The *Bacteroides* genus is not limited to the intestinal tract; in fact, organisms resembling this group have been isolated from other sources as, for example, the respiratory passages (present authors), and inflammatory lesions (Henthorne, Thompson and Beaver, 1936).

In differentiating the *Bacteroides* from the *Fusobacterium* group some difficulty is encountered. This confusion is more apparent than real and is due largely to faulty description. For the most part, the fusiform group is more rapid in its growth, and the cells are longer than those of the known *Bacteroides;* furthermore, they often possess tapering ends, and distinct granules. The fusiform group of bacteria requires highly specialized media. There are, however, some so-called borderline types which in certain respects resemble both the *Bacteroides* and *Fusobacterium* genera.

## MORPHOLOGY

The Gram-negative *Bacteroides* vary rather markedly in their morphology, according to their age and the type of medium upon which they are grown. They are small rods, as a rule. When grown on blood agar, the cells vary from 0.3 to 0.7$\mu$ in thickness, and from 0.5 to 3.0$\mu$ in length. There are, of course, exceptions to the rule. Some *Bacteroides* members may even become filamentous at times. All of the strains studied here were nonmotile.

As will be shown later, morphological characteristics agree fairly well with each other and supply a logical basis for dividing these organisms into several well-defined sub-groups or species.

The majority of the strains appear as solid-staining rods after 4 days incubation at 37°C. on blood agar. Some may show bipolar staining, and many appear quite granular. Most of the strains are ovoid in cell form, and some become quite coccoid, so that it is often difficult to identify them as rods.

Growths on glucose agar usually reveal a distorted morphology. Growth in broth of any kind leads to material departure from the so-called normal cell form and stimulates the development of long filamentous or large oval forms which contain granules and vacuoles.

The cells may occur single or in pairs; often they are grouped in clusters. Chain formation may be observed.

### COLONY MORPHOLOGY

The colonies on blood agar plates may vary from pin points up to 3 to 4 mm. in diameter. The average size may be given as 1 to 3 mm.

There are several types of colonies, the most common appearing soft, grayish, elevated and opaque, and varying from 1 to 3 mm. in diameter. A second type of colony is grayish but rather transparent, while still another is the pin-point type of colony, inoculum from which usually refuses to grow on glucose agar.

The colonies vary in consistency. Some are moist, others quite dry in appearance; some are mucoid, and others distinctly soft. Nearly all of the colonies have smooth edges; a few strains produced an undulating edge. Most colonies are round and elevated or "dew drop" like in appearance; some may assume peculiar shapes such as rising to a point or peak. Some strains exhibit marked hemolysis on blood agar, but this reaction is not observed often.

### BIOCHEMICAL PROPERTIES

All of the strains grow fairly well and retain their viability in egg-meat, which is used as the regular stock culture medium. Nearly all develop in infusion, or better still, in glucose infusion broth. The type of growth is usually diffuse. Acid is formed in the glucose broth, depressing the pH to around 5.0 to 5.4.

A majority of the strains acidify and coagulate litmus milk. Most of them liquefy gelatin after 30 days at 37.5°C. Indol is formed by a few strains. Lead acetate is frequently blackened, but at no time has reduction of nitrates to nitrites been noted.

### FERMENTATION PROPERTIES

Twenty fermentable substances were employed in these tests. The results of Eggerth and Gagnon were confirmed to a fair degree. The monosaccharides were utilized almost universally, as was lactose. Almost all of the other test substances were

attacked in varying degrees by the different strains. Sorbitol and glycerol were utilized only occasionally, and dulcitol and inositol were never fermented. Our results were at variance with those of Eggerth and Gagnon, in a few instances, especially when small amounts of serum were added to the fermentation medium.

Eggerth and Gagnon used the fermentation tests as a basis for classification. After evaluating our results, and after noting the differences between them and those of Eggerth and Gagnon, we were forced to conclude that a classification based largely on such reactions is illogical. It must be admitted that the fermentation properties of individual strains vary over different periods of time and under even slightly different environmental conditions. Indeed, on studying our results and attempting to correlate them with the serological grouping no evidence was found in favor of any classification based on fermentation reactions. For this important reason the fermentation results are not presented here in full—in spite of the fact that the group as a whole is active, fermentatively, especially in some media as, for example, deep agar tubes.

## PATHOGENICITY

Various suspensions of the different *Bacteroides* cultures were injected into white mice, guinea pigs and rabbits by the intraperitoneal and intravenous routes. In two instances the animals died after the injections, but subsequent injections of the same strains did not confirm the original results. From the combined results in these experiments we may safely conclude that the organisms employed are non-pathogenic for mice, guinea pigs and rabbits. Whether these organisms may assume a pathogenic or otherwise harmful (or beneficial) rôle in the intestine, their natural habitat, is at best only problematical.

## SEROLOGICAL PROPERTIES

Eggerth and Gagnon (1933) stated that they had not succeeded in their attempts to develop agglutinogenic antisera for the *Bacteroides* group.

Assuming at this stage of the investigation that serology offered

our main, if not only, hope of classification, we undertook to produce several antisera. The procedure involved the use of large and heavy inoculums freshly prepared from blood agar plates. The bacterial cells were, after 5 or 6 washings, injected into rabbits. Each of the animals received at least 16 injections, with the result that 10 antisera of a fairly satisfactory titer were developed. Most of the sera had agglutination titers of 1:320, and several as high as 1:10,240. Moreover, the sera did not appear to be strain-specific, and promised to offer opportunity for a logical classification of the Gram-negative members of the genus.

Antigens were prepared with washed cells from blood agar plates. The agglutination tubes were set up in the usual manner and examined after 24 hours holding at 37°C. Additional readings were made after further holding for 24 hours, and after 48 hours at room temperature. One antiserum showed a pro-zone reaction.

## PROPOSED CLASSIFICATION

Rather than postulating, as Eggerth and Gagnon suggested, 18 species in this group, and these based almost wholly on fermentation reactions, we propose a simpler classification founded largely on morphological and serological data. The wisdom of adopting such a plan, and its permanency, can be determined only after much more extensive taxonomic research in this field. The classification proposed by Bergey (1934) and based on reactions in litmus milk in no way harmonizes with the data obtained by us and, in our judgment, rests on faulty premises.

*Proposed classification based chiefly on morphology and agglutination reactions*

All anaerobic, non-spore-forming non-motile Gram-negative rods.

Group I. (The most common division of the *Bacteroides* genus)

*Type strain* and proposed name: *Bacteroides vulgatus* (Eggerth and Gagnon).

*Cell morphology:* Ovoid, solid-staining rods, occasionally bipolar-staining, appearing either single or in pairs. Sometimes grouped. Average size 0.5 to 0.7 × 1 to 1.5$\mu$.

*Colonies:* On blood agar usually elevated, grayish and opaque with smooth edges. Average diameter 1 to 3 mm. Glucose agar colonies usually smaller, but growth fairly good.

*Infusion broth:* Growth diffuse and usually heavy; pH of glucose broth culture between 4.8 and 5.4, most strains about 5.0.

*Litmus milk:* Usually acidified, without coagulation.

*Indol:* Variable, most strains negative.

*Gelatin:* Variable, most strains liquefying.

*Lead acetate:* Positive.

*Fermentations:* Variable.

Nitrates not reduced.

Non-pathogenic for mice, guinea pigs and rabbits.

Serologically quite well grouped, although there are a few exceptions.

*Optimum temperature:* 37.5 C.

*Habitat:* Intestinal tract.

Of the strains named and described by Eggerth and Gagnon, the following fall into group I:

*Bacteroides vulgatus*
*Bacteroides insolitus*
*Bacteroides incommunis*
*Bacteroides convexus*

While the following organisms were not available for study, they probably fall into the same division (group I):

*Bacteroides thetaiotaomicron*
*Bacteroides uniformis*
*Bacteroides distasonis*
*Bacteroides tumidus*
*Bacteroides ovatus*
*Bacteroides siccus*

Group II. (Another prominent division of the genus *Bacteroides*)

*Type strain* and proposed name: *Bacteroides varius* (Eggerth and Gagnon).

*Cell morphology:* Granular staining oval bacillus, 0.5 × 0.5 to 1$\mu$. Organisms usually appear in clusters, or grouped in pairs, occasionally short chains.

*Colonies:* On blood agar usually gray, elevated, entire, 2 to 3 mm. in diameter. They vary from opaque or slightly opaque to trans-

parent. Pin point colonies may appear. This group does not grow as well as group I on glucose agar.

*Infusion broth:* Growth in plain and glucose broth usually heavy and diffuse. Final pH in glucose broth around 4.8.

*Litmus milk:* Usually acidified and coagulated.

*Indol:* Positive.

*Gelatin:* Variable, mostly liquefying.

*Lead acetate:* Positive.

*Fermentations:* Variable

Nitrates not reduced.

Non-pathogenic for mice, guinea pigs and rabbits.

Anaerobic, non-spore forming, non-motile, Gram-negative rods.

Serologically quite well grouped.

*Optimum temperature:* 37.5°C.

*Habitat:* Intestinal tract.

Of the strains named and described by Eggerth and Gagnon, the following belong in this group:

*Bacteroides gulosus*
*Bacteroides varius*

The group should probably also include:

*Bacteroides coagulans*

Group III. (An uncommon member of the genus)

*Type strain* and proposed name: *Bacteroides uncatus* (Eggerth and Gagnon). This group seems to be set apart from all of the others by agglutination, morphology and cultural reactions.

*Cell morphology:* Slender pointed bacilli; curved—hooked forms present. Vary from 0.5$\mu$ to distinct filamentous form. Average size 1 to 3$\mu$. They appear to be closely related to the *Fusiformis* genus, morphologically.

*Colonies:* On blood agar colonies are usually very small or pin point. Glucose agar generally shows no growth.

*Infusion broth:* Growth is diffuse, but not nearly as heavy as that of the other groups. The pH of glucose broth varies between 5.6 and 6.0, never falling below 5.6.

*Litmus milk:* Variable.

*Indol:* Negative.

*Gelatin:* Positive (liquefying).

*Lead acetate:* Negative. The only organisms which do not brown lead acetate fall in group III.

*Nitrate:* Not reduced.

*Non-pathogenic* for mice, guinea pigs and rabbits.

*Serologically* well grouped.

*Optimum temperature:* 37.5°C.

*Habitat:* Intestinal tract.

Of the strains of Eggerth and Gagnon, the following belong in this group (III):

*Bacteroides uncatus*
*Bacteroides vescus*
*Bacteroides exiguus*

### Group IV

In many classification schemes there is a so-called waste-basket group. Group IV constitutes such a group here; it is made up of organisms which agree morphologically and culturally with some other group, but fail to be related to the same groups serologically.

Of the strains described by Eggerth and Gagnon there are two which should fall into group II morphologically and culturally, but which show no serological relationship to this group, or to any other group, or to each other. These strains are:

*Bacteroides variabilis*
*Bacteroides inaequalis*

## DISCUSSION

As stated before, the above classification is based primarily on agglutination properties, and secondarily on morphology. The differentiation of the groups is, on the whole, quite clear-cut; however, there are certain exceptions. Several of the strains studied belong definitely to group I, morphologically and serologically; nevertheless, they cross-agglutinate with group II serum, although not in as high dilutions as with their own. Group III is distinct both morphologically and serologically from the others. Group IV, as has been stated, shows no serological relationship to any other group, but does resemble group II morphologically.

It would be possible to further subdivide groups I and II on

the basis of certain cultural and biochemical characteristics, especially the reactions in gelatin and the production of indol. We do not feel justified, however, in making such a division, in view of the fact that the serological results would not support it.

Detailed descriptions of the organisms are not given here, since they would require more than the permissible amount of Journal space.

## CONCLUSION

The Gram-negative members of the *Bacteroides* genus are the predominant organisms in the intestine of most human adults. This observation is in agreement with those of Eggerth and Gagnon.

The Gram-positive members of the group are quite distinct from the Gram-negative, and should be considered apart from the latter.

Our morphological and cultural studies are, on the whole, in agreement with those of Eggerth and Gagnon. We do not believe, however, that a final classification should be based on them alone.

A classification of the Gram-negative organisms into 4 groups based primarily on serology, and secondarily on morphology, is proposed here. Such a classification is in the direction of greater simplicity.

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